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VIROLOGY

Virology 311 (2003) 89–95

www.elsevier.com/locate/yviro

Differential transgene expression in brain cells in vivo and in vitro from AAV-2 vectors with small transcriptional control units

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Received 11 October 2002; returned to author for revision 9 December 2002; accepted 16 January 2003

Abstract

Adeno-associated- (AAV) based vectors are promising tools for gene therapy applications in several organs, including the brain, but are limited by their small genome size. Two short promoters, the human synapsin 1 gene promoter (hSYN) and the murine cytomegalovirus immediate early promoter (mCMV), were evaluated in bicistronic AAV-2 vectors for their expression profiles in cultured primary brain cells and in the rat brain. Whereas transgene expression from the hSYN promoter was exclusively neuronal, the murine CMV promoter targeted expression mainly to astrocytes in vitro and showed weak transgene expression in vivo in retinal and cortical neurons, but strong expression in thalamic neurons. We propose that neuron specific transgene expression in combination with enhanced transgene capacity will further substantially improve AAV based vector technology.

Introduction

Gene therapy vectors based on recombinant adeno-associated virus (AAV; Parvoviridae, Parvovirinae, Dependovirus) have become widely accepted for transduction of the brain. They show neither inherent vector toxicity nor immunogenicity and are not associated with any known human disease. Long-lasting transgene expression from the recombinant genome, which is devoid of all viral genes except the short inverted terminal repeats (ITRs), is a further benefit (Peel and Klein, 2000). Recently developed improvements for production of recombinant vectors (Grimm et al., 1998; Zolothukin et al., 1999) may soon allow for clinical trials using AAV based vectors for the treatment of challenging diseases such as Parkinson's syndrome (Bjorklund et al., 2000). The main issue of AAV based vectors is the relatively small genome size which must not substantially exceed 5000 bp, including 300 bp for the ITRs (Dong et al., 1996). In order to retain sufficient capacity for one or more transgenes (e.g., a therapeutic *and* a reporter gene) transcriptional control elements should be as small as possible. This can be achieved by the use of ubiquitously active viral promoters such as the human cytomegalovirus (hCMV) or the SV-40 promoter. However, forthcoming gene therapy applications will require cellular promoters restricting transgene expression to defined tissues as an important safety criterion. So far, cellular promoters used to drive efficient transgene expression in the brain from AAV vectors were relatively bulky promoters like the neuron specific enolase (NSE) promoter (1800 bp), the hybrid CMV/chicken β-actin (CBA) promoter (1700 bp), or the platelet-derived growth factor-β chain promoter (1500 bp) (Paterna et al., 2000; Björklund et al., 2000; Xu et al., 2001).

The small human synapsin 1 gene promoter (hSYN, 480 bp) has recently been shown to target transgene expression from adenoviral vectors exclusively to neurons in vitro and in vivo, although the tropism of adenoviral vectors favour glial transduction (Kügler et al., 2001; Glover et al., 2002). Furthermore, this promoter showed long-term transgene expression from an adenoviral vector in the rat brain in vivo (Kügler et al., 2002). Since the hSYN promoter is only 480 bp in length we supposed that it would be ideally suited to drive transgene expression from AAV vectors in the brain.

As another small promoter that has not been tested in AAV vectors before, we investigated the murine cytomeg-

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doi:10.1016/S0042-6822(03)00162-4

alovirus (mCMV) immediate early promoter (527 bp). The mCMV immediate early promoter has been demonstrated to be extremely powerful when used in adenoviral vectors (Addison et al., 1997; Gerdes et al., 2000). We therefore aimed to evaluate the expression characteristics of this promoter in an AAV-2 vector, because this virus has been reported to have a neuron restricted tropism (Bartlett et al., 1998). We intended to determine whether the ubiquitously active mCMV promoter, which after adenoviral transduction of the brain is highly active in glial cells, may be "retargeted" to neurons by a vector with preferential neuronal tropism.

By using bicistronic AAV-2 vectors expressing two transgenes (a "functional" transgene plus a reporter gene) contemporaneously from either the hSYN or the mCMV promoter, we demonstrate that (1) the hSYN promoter conferred exclusive neuronal expression in vitro and in vivo; (2) the murine CMV promoter showed diverse expression profiles in the brain, with high activity in thalamic neurons but very weak activity in cortical and retinal neurons; (3) unlike in the adult rat brain, AAV-2 vectors efficiently transduced glial cells in vitro if the transgene is under control of the mCMV promoter; and (4) small transcriptional control units allow for the efficient expression of two transgenes from AAV-2 vectors under control of a cellular promoter.

Results

Two bicistronic recombinant AAV-2 vectors were constructed: AAV-6p1 expressed Bcl-X_L (tagged with the FLAG epitope) under control of the human synapsin 1 gene (hSYN) promoter and, in a separate expression cassette, EGFP likewise from the hSYN promoter. AAV-8a2 expressed Bcl-X_L from the hSYN promoter but expressed EGFP under control of the murine cytomegalovirus (mCMV) promoter. The Bcl-X_L expression cassette contained a small chimeric intron and the SV-40 polyadenylation site, whereas the EGFP expression cassette contained the woodchuck hepatitis virus posttranslational control element (WPRE) and the bGH polyadenylation site (Fig. 1a). In order to evaluate the general expression characteristics of the hSYN and the mCMV promoters, cultures of primary hippocampal neurons and glial cells were transduced and EGFP expression was monitored over time in situ (Fig. 1b–n). We found that the mCMV promoter mediated stronger EGFP expression during the first 3–5 days after transduction (Fig. 1b–j) but the hSYN promoter caught up at about 5–7 days after transduction (Fig. 1h–n). Strikingly, mCMV- and hSYN-driven EGFP expression obviously took place in different cell types, the former expressing mainly in cells with glial morphology and the latter being clearly restricted to neurons. To clarify this issue, transduced cultures were stained with antibodies for either the neuron specific antigen NeuN or the astrocyte specific antigen GFAP. Although hSYN-driven EGFP expression virtually exclusively colocalized to NeuN immunoreactivity (Fig. 2a–c), we found that mCMV-driven EGFP expression colocalized to NeuN immunoreactivity only in a few cells (Fig. 2d–f) but instead showed colocalization with the astrocyte marker GFAP (Fig. 2g–i).

We also investigated whether both the Bcl-X_L and the EGFP transgenes were properly expressed from both vectors. Since cultured hippocampal neurons endogenously expressed substantial amounts of Bcl-X_L (not shown) we used the FLAG epitope tag to detect Bcl-X_L expressed from the viral vectors in vitro. After AAV-6p1 transduction, FLAG immunoreactivity was detected in most neurons that also expressed EGFP, although the total number of FLAG-positive cells was reduced by about 25% as compared with the number of EGFP-expressing cells (Fig. 2k–m). After AAV-8a2 transduction, EGFP expression mainly took place in cells that showed no FLAG immunoreactivity, indicating that with this vector mCMV driven EGFP expression mainly occurred in glial cells (astrocytes) whereas hSYN driven Bcl-X_L expression occurred in neurons (Fig. 2n–p).

The unexpected expression properties of the mCMV promoter were further investigated in vivo by transduction of the adult rat retina and brain. In the intraocularly transduced retina we found that EGFP expression from the hSYN promoter was highly retinal ganglion cell specific, as demonstrated by colocalization with the retrograde tracer FluoroGold (Fig. 3a–c). Only few cells of the inner nuclear layer were also transduced. By immunofluorescent staining with an Bcl-X antibody we found virtually complete colocalization of EGFP fluorescence and Bcl-X_L expression in retinal ganglion cells after transduction with AAV-6p1 (Fig. 3d). Bcl-X_L expression was also detected specifically in retinal ganglion cells (RGCs) after transduction with AAV-8a2, which expressed this transgene from the hSYN promoter; however, EGFP expression driven by the mCMV promoter was barely detectable in RGCs, whereas some Muller glia cells showed pronounced EGFP fluorescence (Fig. 3e and f).

After vector injection into the brain parenchyma, EGFP expression was completely restricted to neurons when driven by the hSYN promoter as revealed by colocalization with the neuron specific antigen, NeuN (Fig. 3g–j). The mCMV promoter, however, showed diverse expression characteristics: in the motorcortex strong EGFP expression was detected in a small number of astrocytes (as identified with GFAP immunostaining, not shown), whereas expression in neurons was very weak (Fig. 3k and l). In contrast, after transduction of the thalamus equal levels of EGFP expression were detected from both the mCMV and the hSYN promoters (Fig. 3m and n).

When we compared the number of cells expressing either EGFP or Bcl-X_L after AAV-6p1 transduction in the striatum, we found that in accordance with the results obtained in vitro, about 30 $\pm$ 4% of EGFP-expressing cells did not express Bcl-X_L. These results indicated that the Bcl-X_L

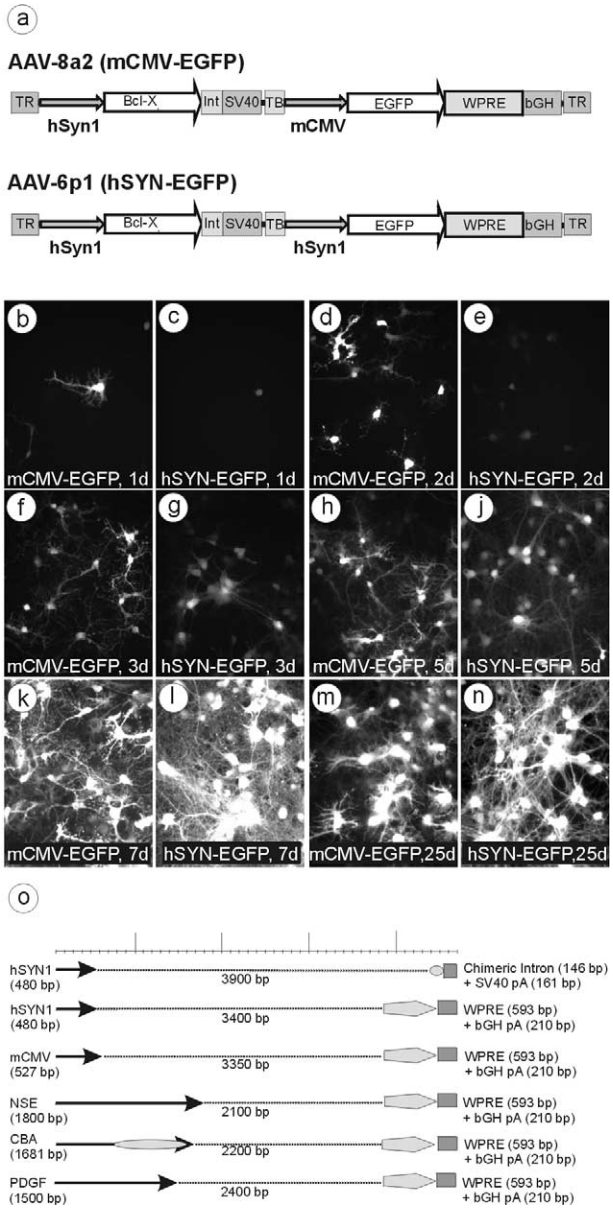

Fig. 1. Expression kinetics of the hSYN and mCMV promoters in cultured primary brain cells in vitro. (a) The recombinant genomes of the two bicistronic AAV-2 vectors. Abbreviations: TR, inverted terminal repeats; hSYN1, human synapsin 1 gene promoter; Bcl-X_L, cDNA sequence coding for the rat Bcl-X_L protein; Int, chimeric intron (from the pCI-Neo vector, Promega); SV-40, simian virus-40-derived polyadenylation site; TB, synthetic transcription blocker; mCMV, murine cytomegalovirus promoter; EGFP, cDNA coding for enhanced green fluorescent protein; WPRE, woodchuck hepatitis virus posttranscriptional control element; bGH, bovine growth hormone gene derived polyadenylation site. Evaluation of the basic expression kinetics from the hSYN and mCMV promoters, respectively, was performed by transduction of primary hippocampal cultures and recording of EGFP fluorescence of live cultures at the specified points of time (b and c = 1 day; d and e = 2 days; f and g = 3 days; h and j = 5 days; k and l = 7 days; m and n = 25 days after transduction). (b, d, f, h, k, m) Transduction with AAV-8a2 (mCMV-EGFP); (c, e, g, j, l, n) transduction with AAV-6p1 (hSYN-EGFP). (o) the genome sizes occupied by different currently used transcriptional control elements are shown. The scale denotes the cloning capacity of AAV-2 based vectors (4.7 kb). The stippled line between promoters and 3′-regulatory elements points up the residual capacity for transgenes to be inserted into the vector. Ellipsoids denote introns, pentagons denote the WPRE, and rectangles denote polyadenylation sites.

expression cassette, which contained the small intron and SV40 polyadenylation site, is somewhat less effective than the EGFP expression cassette that contained the WPRE and bGH polyadenylation site (Fig. 3o–r).

Discussion

AAV-based vectors are rapidly emerging tools for gene therapy applications and many significant improvements regarding vector production, purification, and enhanced transgene expression have been accomplished. Downsizing transcriptional control elements allows for enhanced transgene capacities, and the use of cellular promoters, which show highly cell-type-restricted activity, adds an important safety feature to the vector. Here we demonstrated for the first time that the small human synapsin 1 gene promoter is suited for gene transfer applications in the brain using AAV vectors. As well as in adenoviral vectors, the hSYN promoter mediated completely neuron-restricted transgene expression in both the adult brain and cultured embryonic neurons. Because vectors based on the AAV-2 capsid show a high degree of neuronal tropism, the main advantage of using the hSYN promoter in this type of vector is the gain in transgene capacity (Fig. 1o). We were able to express both a putative “functional” transgene (Bcl-X_L) and the fluorescent reporter EGFP independently under control of cellular promoters. This strategy is promising, especially for retinal transduction since transgene expression can be monitored in the living animal by fluorescent imaging (Rabinowitz et al., 2002). As retinal ganglion cells are the target in a well-established axonal lesion model (Kügler et al., 1999; Kermer et al., 2001), the specific transduction of this cell type by AAV-2 vectors further strengthened the usability of this vector for neurotraumatic research. The vector system described in this study combines small but absolutely neuron-specific transcription control elements with highly retinal ganglion cell-specific transduction. It will allow for the long-term expression of various putatively neuroprotective proteins in retinal ganglion cells in the axotomy model of neurotrauma in order to determine the most effective strategy to overcome neuronal degeneration after traumatic central nervous system injuries. Notably, the choice of an appropriate promoter is crucial to the success of such experiments, as determined by the unexpected failure of the murine CMV immediate-early promoter to mediate transgene expression in retinal ganglion cells irrespective of the efficient infection of these neurons by the AAV-2 virus particle. If possible, expression cassettes should contain the WPRE element, as we have found both in vitro and in vivo that about 30% more cells show transgene expression if this element rather than the chimeric intron was present. However, this difference was much less dramatic than that reported by others (Paterna et al., 2000), indicating that the much smaller intron/SV-40 polyadenylation site mediated efficient transgene expression as well.

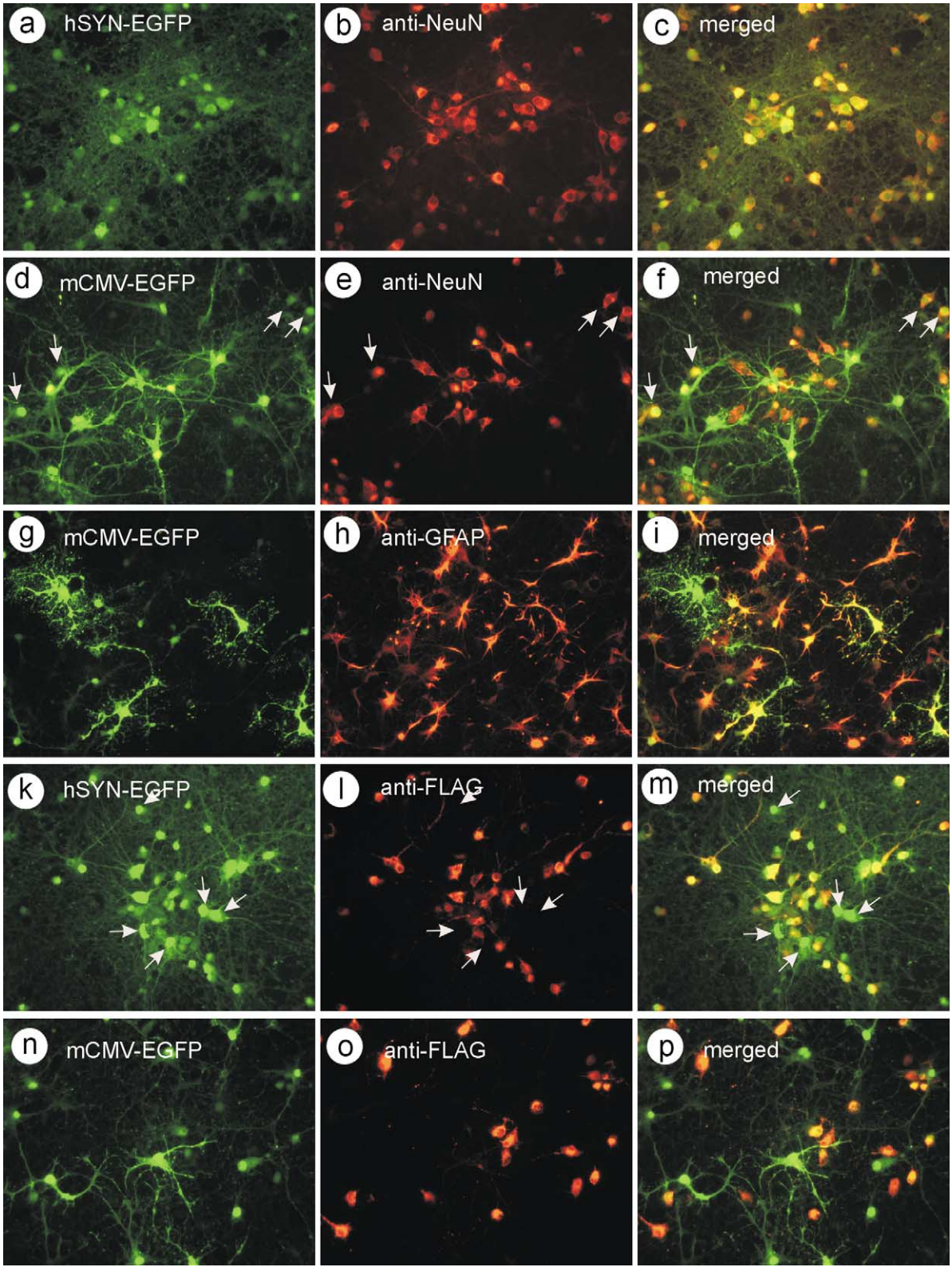

Fig. 2. Transduction properties of AAV-2 vectors with hSYN and mCMV promoters in cultures of primary brain cells. Transductions with AAV-6p1 (expression cassette: hSYN-Bcl-X_L and hSYN-EGFP) and AAV-8a2 (expression cassette: hSYN-Bcl-X_L and mCMV-EGFP) were performed in cocultures of hippocampal neurons and glial cells that were prepared from embryonic brains (E18) and seeded on coverslips. Cultures were transduced at day in vitro 5 with a multiplicity of infection of 1000 and were fixed and stained with the respective antibodies at 10 days after transduction. The left column of the figure (a, d, g, k, and n) shows the EGFP fluorescence obtained from either the hSYN (AAV-6p1) or the mCMV (AAV-8a2) promoter. The center column (b, e, h, l, and o) shows immunoreactivity for the neuronal antigen NeuN (b and e), the astrocyte antigen GFAP (h), and the epitope tag FLAG (l and o; incorporated in the Bcl-X_L transgene, which both vectors express under control of the hSYN promoter). A Cy3-labeled secondary antibody was used for visualization. The right column (c, f, j, m, and p) shows double exposures for EGFP and the respective immunoreactivity. Arrows in (d–f) point to a subset of neurons which show EGFP expression after AAV-8a2 (mCMV-EGFP) transduction. (k–m) Arrows point to neurons which express EGFP but not FLAG tagged Bcl-X_L.

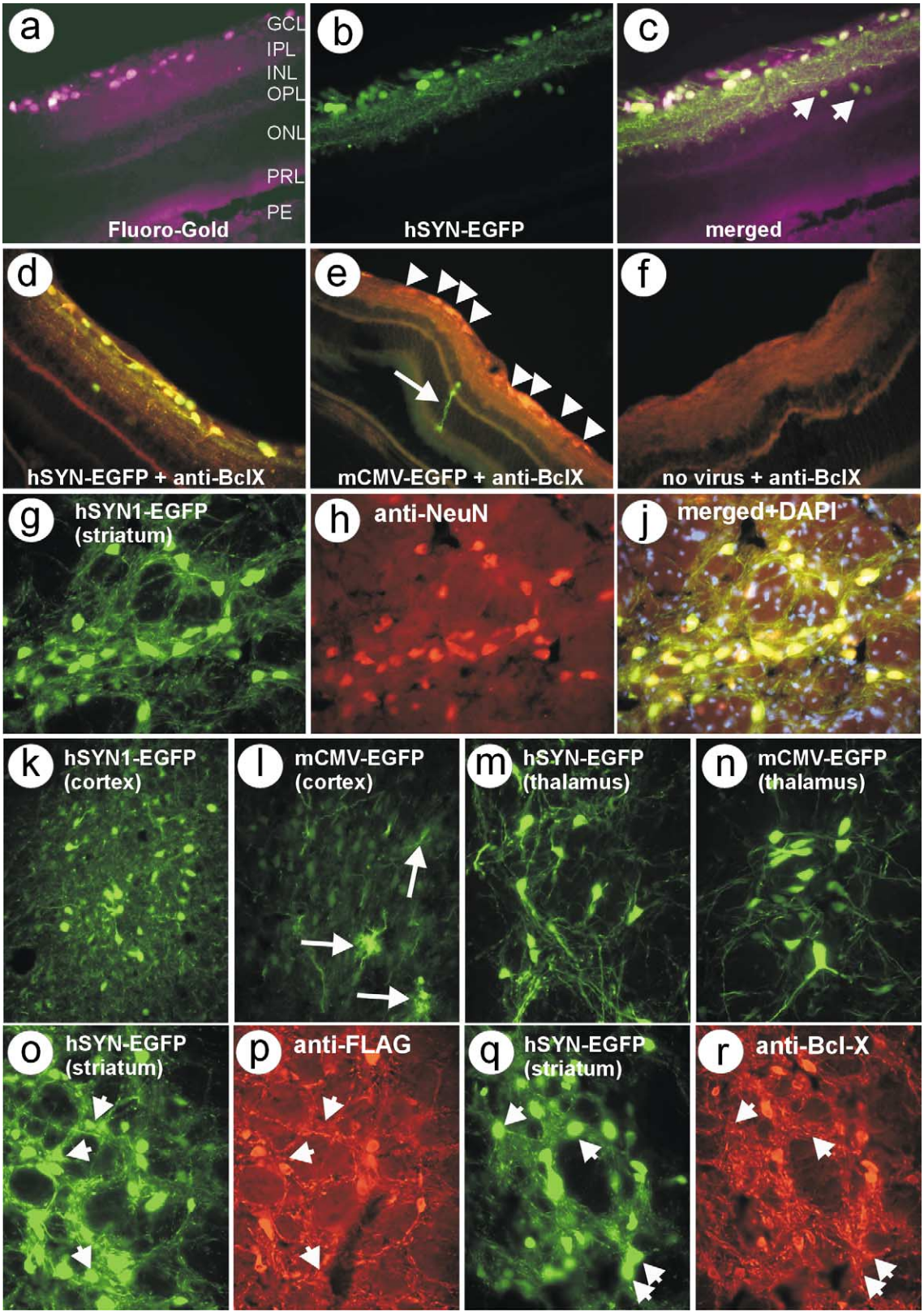

Fig. 3. Transduction properties of AAV-2 vectors with hSYN and mCMV promoters in the adult rat retina and brain. Transductions with AAV-6p1 (expression cassette: hSYN-Bcl-X_L and hSYN-EGFP) and AAV-8a2 (expression cassette: hSYN-Bcl-X_L and mCMV-EGFP) were performed in the retina (a–f) or brain (g–r) of adult rats. (a) Retinal ganglion cells retrogradely labeled with the fluorescent tracer FluoroGold. Abbreviations: GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer; PRL, photo receptor layer; PE, pigment epithelium). (b) EGFP expression from the hSYN promoter after intravitreal transduction with AAV-6p1. (c) Double exposure of FluoroGold and EGFP

To our knowledge, the murine CMV promoter has not been used in AAV vectors before. We tested this short promoter, because it has been shown that the murine CMV promoter permits for even stronger transgene expression with less species restriction than the commonly used human CMV promoter (Addison et al., 1997). Since AAV vectors are "slow" with respect to the onset of transgene expression due to the time it needs to convert the single-stranded vector genome into transcribeable double-stranded DNA, we expected that a very strong promoter might be useful to achieve faster transgene expression. The neuronal tropism of AAV-2 vectors should thereby target expression to neurons. However, we found that only in the thalamus the mCMV promoter showed activity comparable to the hSYN promoter. In cortical and retinal neurons there was weak, if any, transgene expression from the mCMV promoter but some strong glial expression instead. Strikingly, in cultured brain cells obtained from embryonic hippocampus, the mCMV promoter targeted AAV-2 vectors mainly to astrocytes. There appears to be a pronounced difference in the tropism of AAV-2 vectors in either adult brain or embryonic brain cell culture. Although the human and the murine cytomegaloviruses have an identical tropism, the sequences of their immediate-early promoters show drastic differences for known consensus binding sites of cellular transcription factors (Sandford and Burns, 1996). Therefore, these two promoters may have very different expression kinetics depending on the transduced tissue. After all, the results obtained with the hSYN and the mCMV promoter stress the fact that cellular promoters are superior to viral promoters and further suggest that the short human synapsin 1 gene promoter fragment might be valuable in gene therapy approaches in primates and even humans. The high affinity of AAV-2 based vectors for nigral dopaminergic neurons (Björklund et al., 2000) favors the use of the described vector system in Parkinson's research, making it possible to express either large transgenes or a combination of two transgenes. Monitoring of both spatial and temporal transduction efficacy is critical in models of inhibition of neurological degeneration as well as in attempts to generate genetic models of idiopathic Parkinson's syndrome by means of viral vector-mediated gene expression. The coexpression of a versatile reporter gene (like EGFP or DsRed) together with the transgene under investigation may therefore greatly facilitate quantitative studies. In case of space restrictions of vector genomes internal ribosomal entry sites may be helpful in order to express a combination of transgenes. However, small promoters and polyadenylation sites allowing the expression of two transgenes under control of identical transcriptional control elements should be favorable simply because both transgenes will be expressed independently but with identical kinetics. As mentioned above, this strategy will also be valuable for neurotraumatic research in the retino-tectal projection (axotomy of retinal ganglion cell axons), which serves as an broadly accepted model for traumatic neuronal injury (Bähr, 2000).

Materials and methods

Bicistronic expression cassettes (Bcl-X_L under hSYN promoter control and EGFP under either hSYN promoter or mCMV immediate-early promoter control, Fig. 1a) were cloned into pTR-UF 22, from which all elements in between the ITRs except the WPRE and the bGH polyadenylation site were removed. Recombinant vector was prepared by the University of Florida vector core, using an adenovirus-free protocol (Zolothukin et al., 1999). A genome titer as determined by dot blot were 5.9×10^{12}/ml for AAV-8a2 and 4.3×10^{12}/ml for AAV-6p1. A transducing titer of AAV-8a2 (which contained EGFP under mCMV promoter control) was determined by co-infection with adenovirus on 293 cells and was 6×10^{10} transducing units/ml. Matching transducing titer for AAV-6p1 was determined on cultured hippocampal neurons by staining cells with an anti-FLAG antibody (M2, Stratagene) for the epitope-tagged Bcl-X_L, which in both vectors was expressed under hSYN promoter control. For expression experiments on cultured neurons, vectors were used at a theoretical m.o.i. of 1000 transducing units per cell. Preparation of primary neuronal cultures has been described (de Hoop et al., 1998; Kügler et al., 2001). Retrograde labeling of retinal ganglion cells was performed by stereotaxic injection of the tracer FluoroGold into the superior colliculi 7 days before vector application. Vectors were injected into the temporal retinal side intraocularly by means of a fine glass capillary attached via tubing to a Hamilton syringe (1×10^{10} genomes). Stereotaxic vector applications into the brain ($1–2 \times 10^9$ genomes) were performed according to the coordinates of Paxinos and Watson (1998) into primary motor cortex (B: 1.7, L: 3, DV: 2),

fluorescence; arrows point to few cells which are expressing EGFP but are not retinal ganglion cells. (d) Double exposure of EGFP fluorescence (green) and Bcl-X_L immunoreactivity (red) after intravitreal AAV-6p1 transduction, yellow color denotes coexpression of both transgenes. (e) Double exposure of EGFP fluorescence (green) and Bcl-X_L immunoreactivity (red) after intravitreal AAV-8a2 transduction (arrowheads point to retinal ganglion cells expressing Bcl-X_L from the hSYN promoter, the arrow denotes a Müller glia cell expressing EGFP from the mCMV promoter. (f) Retinal section after mock transduction and Bcl-X_L antibody staining. (g) EGFP expression in striatum after AAV-6pl (hSYN-EGFP) transduction. (h) Identical section as in (g) stained for the neuron specific antigen NeuN. (j) Triple exposure of EGFP fluorescence (green), NeuN immunoreactivity (red), and nuclear DAPI stain (blue) (k) Strong EGFP expression in cortical neurons after AAV-6p1 (hSYN-EGFP) transduction. (l) Weak EGFP expression in cortical neurons after AAV-8a2 (mCMV-EGFP) transduction; arrows point to some glial cells (as identified by colocalization with the astrocyte marker GFAP; not shown) that strongly express EGFP. (m and n) equally strong EGFP expression in thalamic neurons driven by either the hSYN or the mCMV promoter. (o–r) Striatal transduction with AAV-6p1 showing expression of both transgenes, EGFP (o and q) and Bcl-X_L the latter detected by either the FLAG epitope antibody (p) or the Bcl-X antibody (r). Arrows point to striatal neurons which show EGFP expression but not Bcl-X_L expression.

striatum (B: 0.2, L: 3, DV: 5.7), and thalamus/globus pallidus (B: −0.8, L: 2, DV: 7). A 2-μl viral suspension was injected over 5 min into female Wistar rats (260 g body wt) anesthesized with chloralhydrate (420 mg/kg). Brains were perfused at 5 weeks after transduction; cut to 16-μm sections; and stained with anti-NeuN (Chemicon), anti-FLAG (Stratagene), and anti-Bcl-X (Transduction Laboratories). Cy3-labeled secondary antibody was used for visualisation on a Zeiss Axiovert Microscope equipped with a CCD camera.

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